One-Step Enzymatic Synthesis of Blue Pigments from Geniposide for Fabric Dyeing

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Abstract In this study, we describe a one-step chemoenzymatic reaction for the production of natural blue pigments, in which the geniposide from Gardenia extracts is transformed by glycosidases to genipin. Genipin is then allowed to react with amino acids, thereby generating a natural blue pigment. The β-glycosidases, most notably Isolase (a variant of β-glucanase), recombinant β-glucosidase, Cellulase T, and amylases, were shown to hydrolyze geniposide to produce the desired pigments, whereas the α-glycosidases did not. Among the 20 tested amino acids, glycine and tyrosine were associated with the highest dye production yields. The optimal molar ratio of geniposide to glycine, two reactants relevant to pigment production, was unity. The natural blue pigments produced in this study were used to dye cotton, silk, and wool. The color yields of the pigments were determined to be significantly higher than those of other natural dyes. Furthermore, the color fastness properties of these dyes were fairly good, even in the absence of mordant.

Keywords: geniposide, genipin, glycosidase, natural pigments, natural dyes

INTRODUCTION

The fruit of *Gardenia jasminoides* is a plant source from which can be obtained a series of yellow, blue, and red pigments, all of which have been employed in the food, cosmetic, and textile industries [1-3]. The iridoid constituents of the colorless components of Gardenia fruits, which are used as tranquilizers and precursors of gardenia blue and red pigments, are comprised of genipin, genipinic acid, geniposide, and geniposidic acid [4]. Geniposide is the principal iridoid glucoside, and can be transformed into genipin via the activity of β-glycosidases. Genipin can then be allowed to react with amino acids, a process which generates natural blue pigments [5,6]. The entire procedure by which natural blue pigments are produced from geniposide is shown in Fig. 1. The iridoid glucosides have been converted into blue pigments via the action of both enzymes and microorganisms, in the presence of hydrolyzed proteins and amino acids [7-9]. The chemical mechanism by which genipin and primary amines are transformed into blue pigments has yet to be elucidated in detail, and related structural analyses also have yet to be conducted. The properties and toxicological data of these pigments were previously reported in the study conducted by Touyama *et al.* [5,6]. However, there has been, to our knowledge, no study conducted regarding the optimal conditions for the one-step chemoenzymatic transformation of geniposide to blue pigments. The primary objective of this work was to optimize the reaction conditions for the production of blue pigments with geniposide and amino acids, using various glycosidases. Furthermore, for the first time, we have used these natural blue pigments to dye a variety of fabrics, including cotton, silk, and wool. Recently, natural pigments have become increasingly desired, due to their eco-friendly properties and generally favorable health effects.

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MATERIALS AND METHODS

Materials

Gardenia geniposide was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The amino acids were obtained from Sigma (St. Louis, MO, USA). The protein assay kit was purchased from Pierce Co. (Rockford, IL, USA). All other chemicals used in this study were of reagent grade.

Enzyme Preparation

The Isolase used in this study was from the National Enzyme Company (Forsyth, Missouri, USA). The hemicellulase, Cellulase A, and Cellulase T were obtained from

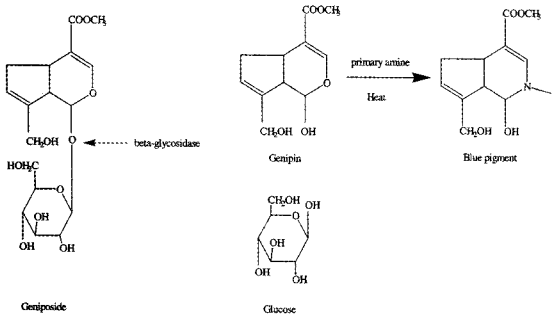

Fig. 1. Blue pigment production from geniposide and amino aicds.

Amano (Nagoya, Japan). The α-amylase and β-glucanase were products of DSM Food Specialties (Charlotte, NC, USA). Barley β-amylase was prepared from Korean barley extract. All other thermophilic enzymes used in the study were from recombinant *E. coli* MV1184 harboring *Thermus caldophilus* enzyme genes [10]. The enzyme genes were obtained from the Glycobiology Lab at KRIBB (Daejeon, Korea). All enzyme activities were assayed via standard protocols.

Blue Pigment Production

The production of pigments using geniposide (1 to 10 mM) and an amino acid (1 to 10 mM) were conducted in 10 mM sodium acetate buffer solution (pH 4.5). Optimal pH and temperature were determined via measurements of pigment yields in a pH range from 4.0 to 12.0 (50 mM of sodium acetate buffer 4.0~5.0, potassium phosphate buffer 6.0~8.0, CAPS buffer 9.0~11.0, Borate/NaOH buffer 12.0) and a temperature range from 25 to 75°C. In order to determine the optimal geniposide:amino acid mole ratio, the reactants were added to sodium acetate buffer (pH 4.5) to a total concentration of 10 mM, and the reaction was conducted at 55°C. The λ_{max} of the blue pigments was determined via scanning from 280 to 700 nm using a UV/visible spectrophotometer (Amersham Biosci. Co., NJ, USA).

Fabric Dyeing with the Natural Blue Pigments

Bleached 100% cotton, silk, and wool fabrics were prepared for our color fastness test by KS K0905. In an attempt to maximize color fastness, the fabric specimens were initially ionized with 0.1% (owf) of INDEX-C, which was obtained from Jungam, Ltd. (Seoul, Korea). The fabrics were then dyed at a 1:20 liquor ratio in separate baths, each containing 4% (owf) of the produced blue pigments. In order to determine optimal dyeing conditions, the dyeing reactions were conducted in four different buffers at various temperatures for several time periods. The pH values were adjusted to 2.2, 3.2, 4.2, and 5.2, using citrate-phosphate buffers. The dyed specimens were thoroughly washed and dried prior to testing. K/S values as a color depth of dyed fabrics were measured on a GretagMacbath Color Eye 3100 (Switzerland). Color fastness relative to water content and perspiration were tested by standard methods: ISO 105-E01 and 105-E04, respectively.

Table 1. Blue pigment production with geniposide and alanine using various enzymes

Enzyme[a]	A_{590}[b]			
	1 h	3 h	5 h	19 h
Isolase	0.73	1.52	1.70	1.70
β-glucosidase	1.04	1.41	1.50	1.60
Cellulase T	0.68	1.36	1.43	1.45
barley β-amylase	0.20	0.40	0.93	1.15
α-amylase	0.10	0.38	0.75	1.10
hemicellulase	0.18	0.26	0.35	0.58
β-glucanase	0.03	0.04	0.04	0.10
Cellulase A	0.03	0.07	0.07	0.09
amylomaltase	0.01	0.03	0.03	0.05
α-glucosidase	0.02	0.03	0.03	0.04

[a]Origin: recombinant β-glucosidase, α-glucosidase and amylomaltase from *T. caldophilus* GK24, Hemicellulase and Cellulase A from *Aspergillus niger*, Cellulase T from *Trichoderma viride*, Isolase from *Trichoderma longibrachiatum*, α-amylase from *Aspergillus* sp., β-glucanase from *bacillus* sp., β-amylase from barley.
[b]Reactants: 1 mM geniposide, 10 mM alanine, 45~70°C.

RESULTS AND DISCUSSION

Selection of Enzyme for the Blue Pigment Production

A variety of glycosidases were tested with regard to their ability to transform geniposide into genipin, resulting in the production of natural blue pigments, via reactions with amino acids. The pigment production yields were summarized in accordance with the enzymes listed in Table 1. Maximum absorbance of the blue pigments was observed at 590 nm when alanine was used as a reactant. The β-glycosidases, in this case Isolase, recombinant β-glucosidase, Cellulase T, and amylases, were shown to hydrolyze geniposide efficiently, thereby effecting a rapid production of blue pigments, whereas the α-glycosidases were ineffective in this regard. Isolase and β-glucosidase were selected as suitable enzymes for the production of the blue pigments, on the basis of reaction rates and production yields.

Effect of Reactants on Blue Pigment Production

Each of 20 amino acids (4 mM) was employed in the production of the natural blue pigments with geniposide by Isolase. The produced pigments evidenced different shades and hues of blue, depending on the amino acids utilized in the reactions (Table 2). The blue pigments with a λ_{max} within a range of 560~580 nm appeared violet, and those in the range of 600~620 nm had a more greenish cast. Among the 20 tested amino acids, glycine, tyrosine, and alanine were shown to be the most effective reactants for the production of the blue pigments.

Table 2. Blue pigment production with geniposide and various amino acids

Amino acid (10 mM)	Enzyme reaction time[a] 3 h		Enzyme reaction time[a] 17 h	
	λ_{max} (nm)	Max. absorbance	λ_{max} (nm)	Max. absorbance
Glycine	570	1.2	570	2.5
Tyrosine	600	1.2	600	2.5
Alanine	592	1.0	585	2.3
Phenylalanine	604	0.6	598	1.8
Glutamine	599	1.0	598	1.7
Methionine	603	0.7	596	1.7
Lysine	580	0.8	580	1.5
Asparagine	608	0.5	598	1.5
Leucine	595	0.5	596	1.5
Valine	594	0.4	594	1.5
Glutamic acid	608	0.4	598	1.4
Arginine	593	0.6	594	1.3
Isoleucine	595	0.4	596	1.3
Aspartic acid	608	0.4	601	1.2
Serine	600	0.5	595	1.1
Tryptophan	604	0.5	604	1.1
Cysteine	615	0.2	595	1.1
Threonine	608	0.4	595	0.9
Histidine	608	0.4	598	0.7
Proline	594	0.2	594	0.7

[a]1 mM geniposide, 50°C.

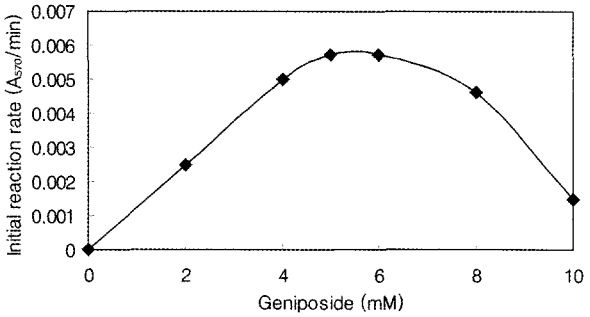

Fig. 2. Effect of the molar ratio of geniposide and glycine on pigment production rates. The total reactant concentrations were 10 mM.

The optimal molar ratio of the two reactants, geniposide and amino acid, was also assessed with regard to the production of blue pigment, with a total reactant concentration of 10 mM. As a result, the optimal molar ratio was determined to be 1:1 (Fig. 2), which indicates that the blue pigments are synthesized using equal molar amounts of genipin and amino acid.

Effects of pH, Temperature, and Enzyme Dosage on Blue Pigment Production

As shown in Fig. 3, the optimal temperature and pH for pigment production appeared to be at a pH of 4.5~6 and a temperature of 55°C, when glycine was used as a reactant. This optimal pH is similar to the optimal pH for

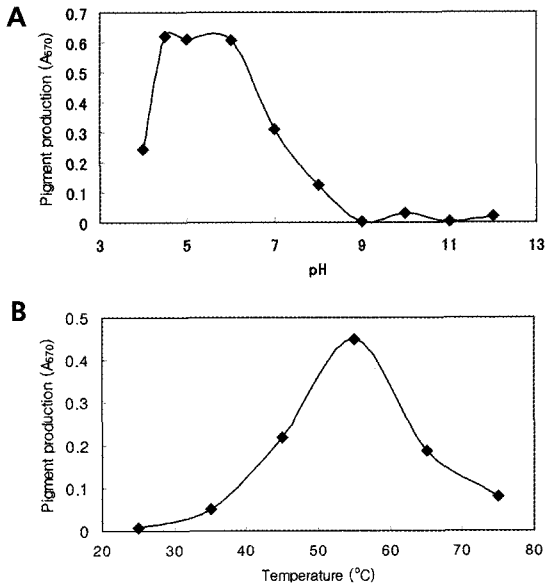

Fig. 3. Effect of pH (A) and temperature (B) on pigment production rate.

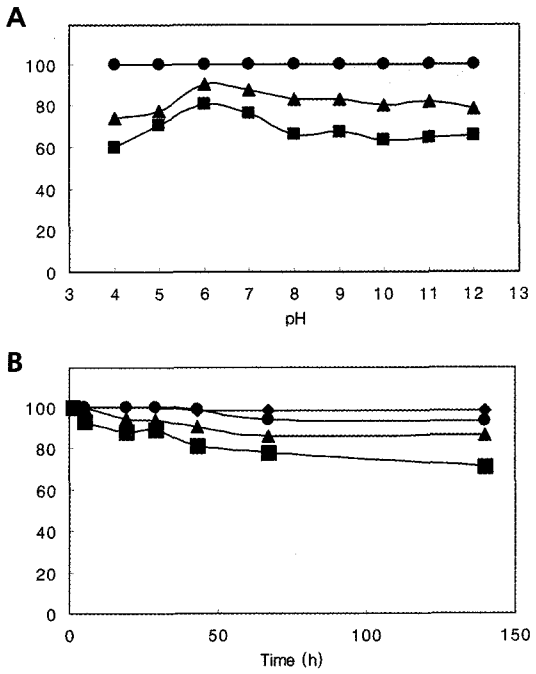

Fig. 4. pH stability (A) and temperature stability (B) of the blue pigments. A_f: final absorbance and A_i: initial absorbance. Symbols: (A) ●, 0 h; ▲, 100 h; ■, 200 h, (B) ◆, 25°C; ●, 30°C; ▲, 55°C; ■, 75°C.

enzyme activity, but the optimal temperature for pigment production is higher than that for enzyme activity, owing to the conjugated reaction of genipin with amino acid.

Pigment production yields were investigated in accordance with the enzyme dosage, using 5 mM geniposide. The production yield was lowered significantly, with an enzyme dosage of below 160 U/g-geniposide, and a dos-

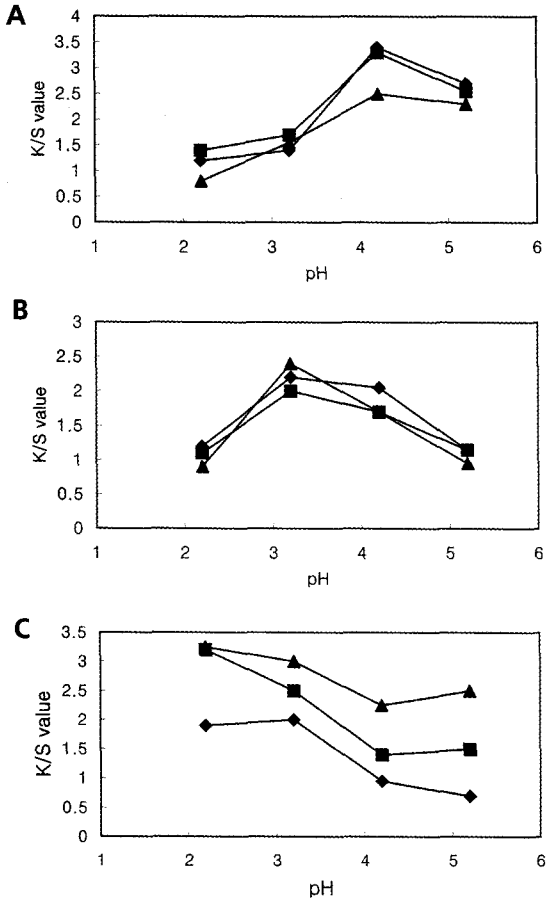

Fig. 5. Color depths of cotton (A), silk (B), and wool (C) dyed with the blue pigments. Treatment time was 30~60 min. Symbols: ◆, 60°C; ■, 70°C; ▲, 80°C.

age of 800 U/g-geniposide was sufficient to obtain a maximum yield (data not shown).

pH and Temperature Stabilities of the Synthesized Blue Pigments

The residual absorbance of the pigments was measured after 200 h of incubation at 55°C in aqueous solutions in a pH range of 4~12, and the results are shown in Fig. 4A. The blue pigments appeared to be unaffected by pH, and retained 80% of initial absorbance after 100 h in a pH range between 5~12.

Temperature stability was assessed in a range of 25~75°C range (Fig. 4B). The absorbance of the pigments was unchanged at 25°C, and remained at 70% of its initial value after 140 h of exposure to a temperature of 75°C. This result indicates that the natural blue pigments are more stable than phycocyanins from blue-green algae.

Dyeing Fabrics Using the Natural Blue Pigments

The color depths of fabrics were affected by three factors; temperature, pH, and dye treatment time. When cotton and silk were dyed with the blue pigments produced in this study, the color depths of the resultant fabrics were influenced significantly by treatment pH (Figs. 5A and B). Our results revealed that the optimal pH and temperature for the dyeing of cotton are 4.2 and 60°C, and 3.2 and 70°C for silk. A treatment time of 30~60 min was determined to be sufficient for the dyeing of each fabric. Color depths were reduced slightly when the fabrics were treated for more than 60 min (data not shown). In the case of the wool dyed with the pigments, color depth was also affected significantly by treatment temperature, as well as by pH (Fig. 5C).

The color fastness characteristics of all three of the fabrics dyed with the blue pigments were fairly good in water. The measured grades were 4~5, which is in accordance with Eco-label limits, and comparable to those of synthetic dyes [11,12]. The grades of color fastness to perspiration were also 4~5 in the silk and wool. However, the color fastness to perspiration grade was as low as 2~3 in the dyed cotton. This means that the blue pigments generated in this study were not sufficiently substantive for the dyeing of cotton without mordants. The sunlight fastness grade was also 2 in both the silk and wool. From a practical perspective, this low degree of substantivity may be improved via the addition of mordants during the dyeing process.

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